Method for Detecting Nucleic Acids with Internal Control of the Amplification

The present invention relates to a method for a qualitative or quantitative detection of a nucleic acid in a sample by means of amplification of this nucleic acid and by using one or several reversibly binding detection probe(s) in which an internal nucleic acid is used that is devised as a control of false-negative results.

The qualitative or quantitative detection of nucleic acid molecules, be it DNA, be it RNA, is an important method of the laboratory medical chemistry especially since PCR-suitable polymerases have been discovered. For this purpose, the target molecules that are present in only a few copies, or even only as a single copy, are amplified and detected by means of probes that can indicate the presence of these molecules.

Such methods must be subjected to certain controls that ensure that the employed amplification system would have enabled the amplification and detection of the molecule to be detected if it had been present in the sample (control of false-negative results, i.e., detection of sample processing efficiency and exclusion of PCR inhibition, for example, by inhibition of polymerase) as well as verify that the employed chemicals have been functional (positive control). Such controls have been prescribed in the meantime in many areas, particularly of laboratory medical chemistry, in the form of standards (see also quality standards in microbiological bacteriological diagnostics MiQ 1/201: nucleic acid amplification techniques).

External controls (occurring in separate reaction chambers) and internal controls (performed in the sample to the examined) must be differentiated. For determining whether the detection test functions properly (positive and negative controls), primarily external control systems have been used in the past. However, in particular for the detection whether an inhibition of the amplification is present it is advantageous to employ an internal control. Internal controls can also be used as quantification standards.

For qualitative as well as quantitative assays, the internal control system is preferably added already at a very early point in time in order to check, if possible, all of the steps carried out in the context of detection of the nucleic acid to be detected.

The internal control is realized in general by addition of an artificial nucleic acid molecule to the sample in which the target nucleic acid (target molecule) is suspected. This artificial molecule corresponds in regard to its base sequence as extensively as possible to the nucleic acid region to be amplified and can therefore be amplified with the same efficiency as the actual target molecule. It is detected in general by employing at least one oligonucleotide detection probe that can hybridize with the target molecule and that carries a reporter group with observable properties that change as a function of whether the probe is bound to the target molecule or is present detached from it. For differentiation from the target molecule, that region of the control nucleic acid molecule that binds to the detection probe(s) is modified relative to the corresponding region of the target molecule. A probe system that is complementary to this region is employed in order to detect the amplified control nucleic acid molecule. In this connection, it is necessary that the second probe system contains a different reporter group than the first one, for example, a dye that absorbs a different wavelength so that it can be detected by a property of this reporter group which property differs from the property to be detected of the first reporter group.

It is desirable that the control system is as similar as possible to the system to be detected in order to obtain a comparability that is as good as possible, particularly for quantitative assays. However, this can entail the disadvantage that as a result of the high homology between the control nucleic acid and the nucleic acid to be detected, these two as well as their amplification products cross-hybridize with one another and therefore cause false results. In EP 1 236 805 A1 a method is described in which a control nucleic acid is used that has a parallel-complementary structure relative to the nucleic acid to be detected. The properties of these

controls, inter alia also e.g. the secondary structures, are particularly similar to those of the nucleic acids to be detected. The controls, however, cannot hybridize with the molecules to be detected so that false results of this kind can be avoided. However, the controls must be detected with probes that are specifically tailored for this purpose. U.S. patent 5,952,202 describes a different detection system with internal control that is based on the detection of reporter/quencher probes that are digested by the employed polymerase during amplification. In this connection, the quenching group is separated from the reporter group and the reporter group, for example, a fluorescent group is activated. An internal control polynucleotide is amplified at the same time with internal control primers and detected by means of a reporter/quencher probe that differs with regard to its spectrometry properties from those of the reporter/quencher probe employed for the detection of the nucleic acid to be detected. In this system, the control nucleotide must be amplified with primers that are specifically tailored for this purpose. A disadvantage of both systems is, on the one hand, that the production of the additional components (particularly the probes) entails costs. Secondly, the detection of a different physical property of the reporter group of this probe(s) requires the use of a second detection system, for example, a separate channel for measuring the absorption/fluorescence at a second wavelength.

It is the object of the present intention to provide a simplified internal control for a nucleic acid detection method in which the nucleic acid is amplified.

This object is solved in that the method is carried out in the presence of a control nucleic acid that is also amplifyable and binds the same detection probe(s) as the nucleic acid to be detected but, in comparison to the nucleic acid to be detected, has deviations in the nucleotide sequence in the binding region for the probe(s) such that the product of control nucleic acid and probe(s) has a different melting point than the product of nucleic acid to be detected and probe(s). The difference of the two melting points must be so great in this connection that the two products can be analytically differentiated by a melting analysis or another detection method.

Expedient in this connection is a value of not less than approximately 5°C. This latter value enables, for example, to differentiate unequivocally the melting temperature of the pair of nucleic acid to be detected and probe(s) from the melting temperature of the pair of control nucleic acid and probe(s) in a melting diagram or the like. An unequivocal detection enables the use of control nucleic acid as an internal control. At smaller temperature differences than the one indicated above it is to be expected in a series of cases that the signals of the bound probes no longer differ from one another so clearly for the control to be considered reliable.

In a preferred embodiment of the invention, the melting point of the product of control nucleic acid and probe(s) is lower and according to the above discussions is especially preferred lower by at least 5°C than that of the product of the nucleic acid to be detected and the probe(s).

According to the invention the same probe system (one or several probes) is used for the detection of the nucleic acid to be detected and the control nucleic acid. On the one hand, this saves costs; on the other hand, the probe(s) for the control nucleic acid must not be detected by means of a second detection system. Moreover, using the present invention eliminates the requirement for performing a positive control that, in turn, controls the controlling probe system in regard to its efficiency. As mentioned above, the internal amplification control according to the invention does not require a second probe system; and, accordingly, a separate control for it is no longer needed.

The present invention is suitable for all probe systems that are not consumed, e.g., digested, during amplification.

Measures for obtaining a suitable melting temperature between control nucleic acid and probe(s) are known to a person skilled in the art. As a general rule, the following binding rule is valid for a well-binding probe (approximately 20 bases): The nucleotides G and C (each having three hydrogen bonds) contribute each

approximately 4°C, the nucleotides A and T (each having two hydrogen bonds) contribute each approximately 2°C to the annealing temperature inasmuch as they are participating in the bonding action (Wallace/Ikutana rule Tm(°C) = 2x(#A+#T) +4x(#G+#C), cited in Wetmur J.G., 1991, Crit. Rev. Biochem. Mol Biol. 26:227-259)). More precise predictions are possible with the so-called "nearest neighbor" method that is based on thermodynamic calculations (Borer P.N. et al., 1974, J. Mol. Biol. 86:843-853, modified by Alawi, H.T. et al., 1997, Biochemistry 36:10581-594). The introduction of a mismatch lowers the melting point usually roughly by 5 to 9°C. In this connection, the type of mismatch has a strong effect on the melting point change. For example, a GT mismatch produces only a very small reduction of the melting point, a CA mismatch the strongest. The effect of the other mismatches is between these two, in particular, in the following sequence GT<GA<TT<GG<AA<CC<CT<CA. In addition, the reduction strongly depends on the position of the mismatch in the binding region and on the base composition in the vicinity of the mismatch. The application of the "nearest neighbor" method on probes that are usable in connection with the so-called LightCycler method (real-time method of the company Roche) and the effect of the mismatches is described in detail in Von Ahsen, N., Schütz, E. (2001), Using the nearest neighbor model for the estimation of matched and mismatched hybridization probe melting points and selection of optimal probes on the LightCycler. In: Rapid cycle real-time PCR, methods and applications, Meuer S., Wittwer, C., Nakagawara, K. (eds), pp. 433-56, Springer Verlag Berlin, Heidelberg, New York.

The above discussion shows that in many cases it will be sufficient to exchange in the nucleotide sequence of the control nucleic acid within the binding region for the detection probe(s) only one nucleotide present in the nucleic acid to be detected for another one. Preferably, at least two deviations are present therein. In order to make the binding of the probe to the control nucleic acid in other respects as comparable to the binding of the probe to the nucleic acid to be detected, it is beneficial in this connection when the mismatches are distributed as uniformly as possible across the binding region.

The method is equally suitable for the detection of different kinds of nucleic acids such as DNA or RNA. For example, it can be used for the detection of pathogen-specific nucleic acid, for example, the detection of viruses, bacteria, fungi, and others. Concrete examples are the detection of different hepatitis viruses, herpes, papilloma viruses, bordetella strains, HIV, corona viruses (triggers SARS).

The control nucleic acid is advantageously added to the sample to be examined as early as possible. It is, for example, expedient to add it already to the fresh sample taken from a patient (blood, urine or the like).

The control nucleic acid is preferably a single-strand nucleic acid, for example, an oligonucleotide, that in the presence of the substances required for amplification of the nucleic acid to be detected is amplified likewise. As an example, and preferred, the invention can be employed in connection with assays that operate based on the well-established PCR amplification technique. In these cases, it is beneficial when the control nucleic acid has such a nucleotide sequence at the 3' end and the 5' end that the amplification of the nucleic acid strand, like the one of the complementary strand, is carried out in the presence of the same primers that are used for the amplification of the nucleic acid to be detected. However, it should be apparent that this preferred embodiment is beneficial because the otherwise required expenditure for the development of detection conditions including the additional components is no longer required and because the complexity of the sample is not increased in this way (additional components increase inter alia the probability of the formation of an undesired primer/primer pair). However, this configuration is not a necessary criterion for the invention.

The number and selection of bases for those regions of the control nucleic acid that are neither required for primer binding nor for the probe binding is of subordinate importance. This is particularly surprising because it has been stressed again and again in the prior art that control nucleic acids should be as similar as possible to the nucleic acids to be detected. And it is advantageous, because the manufacture

of shorter control nucleic acids is significantly less expensive. It was found in accordance with the present invention in contrast to the prior art assumptions that the aforementioned regions of the control nucleic acid often deviate strongly and, especially preferred, they are in a number of cases even significantly shorter, i.e., they can be also deleted largely or even deleted with the exception of a few nucleotides (for example, 1 to approximately 10, up to approximately 20 or up to approximately 30). It is even possible that the control nucleic acid comprises exclusively, or almost exclusively, the sequence regions that hybridize with the probes and the primers and cover them.

Generally, it is not required that the control nucleic acid is amplified in identical copy numbers as long as, on the one hand, an amplification is ensured that enables the reliable detection of the control nucleic acid in the test liquid (sample) and, on the other hand, its amplification is not preferred relative to the amplification of the nucleic acid to be detected in such a way that the latter provides a significantly worsened result. The above described shortening or deviation surprisingly does not lead to one or the other of the aforementioned boundary conditions being surpassed. These conditions, by the way, can be maintained optionally also by controlling the ratio of the employed copy numbers of nucleic acid to be detected and control nucleic acid.

According to the invention, the use of the control nucleic acid is suitable for quantitative as well as qualitative assays of nucleic acid(s). For a qualitative detection, the amplification of the nucleic acid to be detected and that of the control nucleic acid, optionally with the same primers, can be carried out in the desired cycle number. The detection of the nucleic acid to be detected and that of the internal control is carried out in a melting step after amplification. The single-strand amplification product or the amplification product that has been made into a single strand (denatured) is cooled to a temperature at which the probe(s) bind(s) to both nucleic acids. Subsequently, a temperature increase is carried out with detection of the observable properties of the probe system that change because of the

detachment of the probe(s) from the nucleic acid strand. The amplification product hybridized with the probe(s) of the internal control exhibits as a result of mismatched pairs a different, preferably lower, melting point than the amplification product of the nucleic acid to be detected that is hybridized with the probe(s).

In the qualitative as well as in the quantitative determination, the amplification of the control nucleic acid can be carried out "silently" in the background. It is therefore also not decisive that it takes place with the same efficiency as in the case of the nucleic acid to be detected.

The presence of a nucleic acid to be detected in quantitative methods in general is detected at a temperature that is somewhat below its melting temperature with the present probe(s). The melting point of the product of control nucleic acid and of the probe(s) should be selected for quantitative determinations as much as possible such that the two components at this temperature essentially do not hybridize. As a result of this measure, the control system does not interfere with the observation of the nucleic acid to be detected. Optionally, the detection temperature in the method can be selected to be relatively close to the melting temperature of the product of the nucleic acid to be detected and the probe(s) so that the spacing to the melting temperature of probe(s) and control nucleic acid is sufficient.

This is true in particular for the so-called real-time method in which the growth of the nucleic acid to be detected during PCR can be observed, for example, with the so-called LightCycler system of the company Roche. In this system, the probe system is comprised of two probes of which one comprises a fluorescent dye, the other comprises a dye that emits within the visible spectrum. One of the dyes is located at or near the 3' end of the first probe, the other at or near the 5' end of the second probe; the probes each are tailored such that in the state bound to the nucleic acid they are positioned adjacent to one another in such a way that the dyes are in spatial proximity to one another. This leads to a transfer of the fluorescence resonance energy from the fluorescence dye onto the light-absorbing dye (the

reporter group) so that it is excited to emit light. The wavelength of this emission is detected. The annealing temperature and the melting temperature of nucleic acid to be detected and primers or probe system are both below the working temperature of the polymerase. The nucleic acid to be detected is therefore amplified in working cycles that comprise denaturing of the double-strand nucleic acid at a correspondingly high temperature, cooling to the annealing temperature at which the primers bind the probe system and the nucleic acid is detected, and raising the temperature to the working temperature of the polymerase at which the new complementary strand is synthesized onto the nucleic acid.

This detection principle has been performed in the past conventionally in the presence of a control system with a synthetic nucleic acid, primers required for its amplification, and a complementary probe system that is comprised of two probes and comprises a dye that emits at a different wavelength and, in addition, is detected by this second wavelength. The present invention can also be employed in connection with this principle in that, instead, a control nucleic acid is tailored that binds the same probes as the nucleic acid to be detected; however, one or several bases are exchanged in the control nucleic acid such that the melting temperature of the pair of control nucleic acid and probes satisfies the conditions according to the invention described above. In other respects, the probe sterically unchanged still binds to the control nucleic acid so that its reporter group(s) in combination with the control nucleic acid provide the same signal as is delivered by the combination of the probe with the nucleic acid to be detected.

Of course, the above described principle can be employed in an appropriately modified way also to alternative probe systems. An example for this is the system of "molecular beacons". This system operates with only one probe that contains a dye, for example, a fluorescent dye, as well as a quencher group. In the free state, the probe unfolds such that the quencher group is near the dye group and inhibits its emission. In the state bonded to the nucleic acid, the dye group and the quencher group are located at spatially separated parts of the probe that therefore

is fluorescent. In this detection system, an internal control nucleic acid can be used also; it binds the probe in the same way as the nucleic acid to be detected whose pair with the probe however, as a result of mismatches as described above, has a lower melting point. The invention is however applicable also to all other prior art probe systems or probe systems still to be developed which are not consumed during amplification.

The present invention can be employed very well also for so-called multiplex analyses in which several nucleic acids can be detected simultaneously, for example, when searching for certain pathogen variants. Since the control system according to the invention does not require a second detection system, two nucleic acids, for example, can be detected by means of two primer pairs and two probe systems wherein the two probe systems are detected by means of two different reporter groups, for example, by observing two different wavelengths at which these reporter groups emit light. According to the invention, two internal control nucleic acids are added to such a system, each one being tailored as described above in regard to the nucleic acid to be detected. Such a duplex analysis can be performed with conventional two-channel measuring apparatus because no more than the two dyes of the probe systems for the nucleic acids to be assayed must be detected. Accordingly, higher multiplex analyses are possible with multi-channel devices.

In the following the invention will be explained in more detail with the aid of one embodiment that illustrates the use of the control according to the invention with the aid of the detection of the hepatitis B virus.

Hepatitis B DNA is detected by amplification of a section of the gene X comprising 188 base pairs. The section is comprised of the following base sequence (SEQ ID NO. 1)

5'GACGTCCTTTGTTTACGTCCCGTCGGCGCTGAATCCTGCCGACGACCCTT
CTCGGGGCCGCTTGGGGCTCTACGCCCTCTTTGCCGTCTGCCGTTCCAGC
CAACCACGGGGCGCACCTCTCTTTACGCGGTCTCCCCGTCTGTGCCTTCTC

ATCTGCCGGTCCGTGTGCACTTCGCTTCACCTCTGCA3'

This sequence is divided into the following regions:

5'GACGTCCTTTGTTTACGTCCCGTC 3' (1)

5'GGCGCTGAATCCTGCCGAC 3' (2)

5'GACCCTTCTCGGGGCCGCTTGGGGCTCTACGCCCTCTTTGCCGTCTGCC GTTCCAGCCAACC3' (2a)

5'ACGGGGCGCACCTCTCTTTACGCGG 3' (3)

5'T 3' (4)

5'CTCCCCGTCTGTGCCTTCTCATCTGC 3' (5)

5'C 3' (6)

5'GGTCCG 3' (6a)

5'TGTGCACTTCGCTTCACCTCTGCA 3' (7)

The regions 1 and 7 are the primary binding regions. The region 3 refers to the region that hybridizes with the 3' fluorescein-marked probe. The region that hybridizes with the 5'Red640-marked probe is identified by numeral 5. The remaining numerals refer to the remaining sequence regions.

The test is performed in accordance with Jursch et al., Med. Microbiol. Immunol., (Berl), 2002 Mar, 190 (4): 189-197:

F- primer: 5' GAC GTC CTT TGT YTA CGT CCC GTC 3'

R-primer: 5' TGC AGA GGT GAA GCG AAG TCG ACA 3'

anchor probe: 5' ACG GGG CGC ACC TCT CTT TAC GCG G fluorescein 3'

detection probe: 5' LC-Red640 CTC CCC GTC TGT GCC TTC TCA TCT GC Phosphate 3'

The nucleic acid preparation is carried out with the aid of silica membrane columns (company Qiagen) from 200 µl plasma or serum. Elution of the DNA from the column in 50 µl.

PCR batches in glass capillaries of the firm Roche (total volume 10 µl):

5 pmol of each primer and 2 pmol of each probe

1 µl LightCycler FastStart DNA Master Hybridization Probes (contains polymerase and nucleotides)

sample DNA: 2.5 µl.

Operating Conditions in the LightCycler (Roche):

1. DNA denaturization and activation of the polymerase:
   cycles "1"; analysis mode "none"
   segment 1: target temperature "95"; incubation time "10:00"; temperature transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "NONE"
2. precycling program:
   cycles "10"; analysis mode "none"
   segment 1: target temperature "95"; incubation time "10"; temperature transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "NONE"
   segment 2: target temperature "64"; incubation time "8"; temperature transition rate "20.0"; secondary target temperature "54"; step size "1.0"; step delay "0"; acquisition mode "NONE"
   segment 3: target temperature "72"; incubation time "13"; temperature transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "NONE"
3. main cycling program:
   cycles "35"; analysis mode "NONE"
   segment 1: target temperature "95"; incubation time "10"; temperature transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "NONE"
   segment 2: target temperature "54"; incubation time "8"; temperature transition rate "20.0"; secondary target temperature "0"; step size "1.0"; step delay "0"; acquisition mode "NONE"
   segment 3: target temperature "72"; incubation time "13"; temperature transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "SINGLE"

In the main cycling program, the two primers and internally the two probes bind in the so-called annealing step (segment 2: target temperature 54 °C). The green fluorescence dye of the anchor probe (fluorescein) is excited and transmits the energy onto the red dye (LightCycler Red640) of the detection probe (fluorescence resonance energy transfer technology). The red fluorescence that is emitted by the detection probe is measured in every annealing step and corresponds to the increase of the generated PCR product.

Internal control:

For checking the efficiency of the nucleic acid preparation and for excluding false-negative results by inhibition of the PCR reaction, the following oligonucleotide with 120 bases is used as an internal control (SEQ ID NO. 2)

5'GACGTCCTTTGTTTACGTCCCGTCGGCGCTGAATCCTGCCGACACGGGG
CGCACCTCTCTTTACGCGGTCTACCAGTCTATGCCTTATCATCTGCCTGTG
CACTTCGCTTCACCTCTGCA 3'

This sequence is divided into the following regions:

5'GACGTCCTTTGTTTACGTCCCGTC 3' (1")
5'GGCGCTGAATCCTGCCGAC 3' (2")
5'ACGGGGCGCACCTCTCTTTACGCGG 3' (3")
5'T 3' (4")
5'CTACCAGTCTATGCCTTATCATCTGC 3' (5")
5'C 3' (6")
5'TGTGCACTTCGCTTCACCTCTGCA 3' (7")

The primer binding locations are identified by 1" and 7"; they are identical to those of hepatitis DNA. The region identified at 3" that hybridizes with the 3' fluorescein-marked probe is also identical. The region (5") that hybridizes with the 5' Red640-marked probe shows a total of four deviations relative to native hepatitis DNA (four times A in place of C; G; G; C). These deviations concern the third, 6th, 11th, and 18th nucleotide of this region. The regions 2", 4", and 6" represent the remaining sequence region. It corresponds to the regions 2, 4, 6 in the above shown native hepatitis DNA. The regions identified as 2a and 6a are deleted in the control DNA.

An amount of 2.5 µl of the oligonucleotide (concentration 1000 copies/µl) is added to 200 µl plasma or serum. Subsequently, the nucleic acid preparation is performed as described above.

The PCR batches are processed as described above.

Only a minimal modification of the LightCycler operating conditions is required in this connection:

1. DNA denaturization and activation of the polymerase:
   cycles "1"; analysis mode "none"
   segment 1: target temperature "95"; incubation time "10:00"; temperature transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "NONE"
2. precycling program:
   cycles "10"; analysis mode "none"
   segment 1: target temperature "95"; incubation time "10"; temperature transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "NONE"
   segment 2: target temperature "64"; incubation time "8"; temperature transition rate "20.0"; secondary target temperature "54"; step size "1.0"; step delay "0"; acquisition mode "NONE"
   segment 3: target temperature "72"; incubation time "13"; temperature transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "NONE"
3. main cycling program:
   cycles "35"; analysis mode "quantification"
   segment 1: target temperature "95"; incubation time "10"; temperature transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "NONE"
   segment 2: target temperature "54"; incubation time "8"; temperature transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "NONE"
   segment 3: target temperature "60"; incubation time "0"; temperature

transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "SINGLE"
segment 4: target temperature "72"; incubation time "13"; temperature transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "NONE"

4. melting program:
cycles "1"; analysis mode "melting curves"
segment 1: target temperature "95"; incubation time "5"; temperature transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "NONE"
segment 2: target temperature "40"; incubation time "20"; temperature transition rate "20.0"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "NONE"
segment 3: target temperature "85"; incubation time "0"; temperature transition rate "0.20"; secondary target temperature "0"; step size "0.0"; step delay "0"; acquisition mode "CONT"

Modification: raising the fluorescence measuring temperature to 60 °C additional melting program after completion of PCR amplification

The internal control is amplified in the same way as for hepatitis B DNA in the PCR but is not detected during PCR because at the measuring temperature of 60 °C no binding of the detection probe to the internal control takes place.

In Fig. 1 the detection of the pathogen-specific amplification product in the annealing step of PCR is illustrated. At 60 °C both detection probes bind to the pathogen-specific target sequence. Transfer of the fluorescence resonance energy from the fluorescein to Red640 takes place. The red signal is detected. The Figure shows the increase of fluorescence signal during the course of PCR amplification. Curve 1 shows the amplification of 10,000 copies HBV DNA without internal control.

Curve 2 shows the amplification of these copies with control. The curves 3 and 4 are negative controls (without DNA) with (curve 4) and without (curve 3) internal control. As indicated by curve 4, the probes with the internal control do not result in a signal at the selected conditions (measuring temperature 60 °C) because binding of Red640-marked probe is not possible as a result of mismatched pairs. The Figure shows that the addition of internal control has no effect on the result of PCR. The batches with and without internal control show identical results. The internal control is not detected during PCR amplification.

The Internal control can be detected by melting analysis after completion of PCR. In this connection, the amplification product is denatured first at 95 °C. As a result of the subsequent cooling to 40 °C (annealing step) the probes bind to the pathogen-specific amplification product as well as to the amplification product of the internal control even though four mismatches are present. Subsequently, a slow temperature increase with continuous measuring of fluorescence takes place. When melting off the detection probe, a fast drop of the fluorescence signal occurs. Fig. 2 shows the result of the melting analysis. As a result of mismatches, the bonding of the detection probe to the internal control shows a significantly lower melting temperature than the bonding to the hepatitis B PCR amplification product. The melting temperature with internal control was approximately 51 °C, the melting temperature with hepatitis B PCR amplification product was approximately 73 °C.

The Figure shows a diagram with the first derivative of the dye detection. The curve for HBV alone (curve 1) ascends slowly, starting at 45 °C, and shows exclusively one peak at approximately 73 °C. The curve for the HBV test together with the internal control (curve 2) shows two peaks of which the one at approximately 51 °C is caused by the probe melting off the control DNA while the other shows the result of the probe melting off the pathogen DNA. The negative control with internal control (curve 4) shows as expected only a single peak at approximately 51 °C (dashed curve) and the negative control alone (curve 3) shows no fluorescence.